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PRINCIPAL INVESTIGATOR: Jenny Chang, M.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
                          Houston, TX 77030

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6. AUTHOR(S)

Jenny Chang, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Baylor College of Medicine Houston, TX 77030 E-Mail: jchang@breastcenter.tmc.edu	

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13. ABSTRACT (Maximum 200 Words)

Chemotherapy is of proven benefit in reducing the risk of death for a subset of patients with early breast cancer, but doctors have problems deciding exactly who should receive this therapy, and which therapy will be most effective for a given patient. As a result, some patients needlessly receive chemotherapy. Even in those patients who clearly require chemotherapy, doctors cannot identify those patients whose tumors might not be responsive to a particular chemotherapy drug. Chemotherapy is also associated with high costs and toxicity including nausea, vomiting, damage to nerves, etc. and increased risk of infections that are sometimes life threatening. The emerging cDNA array technology provides a means to comprehensively appreciate genetic variations in different breast tumors, and may be utilized as a test for chemotherapy sensitivity. Taxotere has one of the highest response rates in breast cancer, and is widely prescribed for the treatment of breast cancer. The aims of this study were therefore, to investigate and validate differential gene expression patterns from core biopsies from patients whose breast tumors either shrank after Taxotere chemotherapy, or failed to respond. We now have preliminary data that gene expression patterns may predict response and resistance to chemotherapy. These patterns need to be better refined, and validated in subsequent studies. If patterns of gene expression exist that can help better select appropriate therapies, this would enable better selection of appropriate treatments for women with breast cancer.

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Introduction

Optimal systemic treatment (adjuvant therapy) after breast cancer surgery is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients.[1-3] However, while estrogen receptor status predicts for response to hormonal treatments, there are no clinically useful predictive markers for chemotherapy response. All eligible women are therefore treated in the same manner even though *de novo* drug resistance will result in treatment failures in many breast cancer patients. The taxanes, docetaxel (Taxotere™) and paclitaxel (Taxol™), are a new class of anti-microtubule agents that are more effective than older drugs like the anthracyclines,[4-6] although clinical trials with taxanes and anthracyclines in combination show that only a small subset of patients benefit from the addition of taxanes.[7,8] Currently, there are no methods available to distinguish those patients who are likely to respond to taxanes from those who are not, and given the accepted practice of prescribing adjuvant treatment to most patients even if the average expected benefit is low, the *a priori* selection of appropriate patients most likely to benefit from adjuvant taxane therapy would represent a major advance in the clinical management of breast cancer today.[7,8] A major impediment to study predictors of therapeutic efficacy in the adjuvant setting is the lack of surrogate markers for survival and, consequently, large numbers of patients with long-term follow-up are needed to conduct these studies.

We therefore set out to identify gene expression patterns in primary breast cancer specimens that might predict response to taxanes. Neoadjuvant chemotherapy (treatment before primary surgery) allows for sampling of the primary tumor for gene expression analysis, and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment.[9,10] This clinical tumor response to neoadjuvant chemotherapy has been shown to be a valid surrogate marker of survival, with better outcome in those patients whose tumors regress significantly after neoadjuvant chemotherapy compared to those with modest response or clinically obvious chemotherapy-resistant disease.[9,10] With the advent of high-throughput quantitation of gene expression, it is now possible to assess thousands of genes simultaneously to identify expression patterns in different breast cancers that might correlate with and thereby predict excellent clinical response to treatment.[11-15] These profiles have a great potential to penetrate the genetic heterogeneity of this disease and prioritize different treatment strategies based on their likelihood of success in individual patients. Hence, neoadjuvant chemotherapy provides an ideal platform to rapidly discover predictive markers of chemotherapy response. In the present study, we took core needle biopsies of the primary breast cancer for gene expression profiling before patients received neoadjuvant docetaxel. The purpose of this study was 1) to demonstrate that sufficient RNA could be obtained from these core biopsies to assess gene expression, 2) to identify groups of genes that could be used to distinguish primary breast cancers that are responsive or resistant to docetaxel chemotherapy, and 3) to identify gene pathways that could be important in the mechanism of resistance to docetaxel.

Body of Research

From the statement of work, we proposed the following:
Task 1. Core biopsies of 35 patients enrolling in a phase II neoadjuvant Taxotere study from 2 institutions, Baylor/Methodist Breast Care Center and Ben Taub Hospital.
Task 2. Investigation of gene expression patterns in core biopsies of human breast cancers in responders versus non-responders to Taxotere chemotherapy.
Task 3. Validation of gene expression differences by quantitative reverse-transcription polymerase chain reaction (Q-RTPCR), and by immunohistochemistry with antibodies against known proteins encoded by particular genes in the cluster is anticipated time to take 24 months.

Task 1.
From September 17, 2001 to July 2003, we had recruited 65 patients with locally advanced breast cancer. Core biopsies were obtained from the primary breast cancers before commencement of neoadjuvant chemotherapy. Clinical responses before and after four cycles of chemotherapy were measured in all primary breast cancers.

Task 2.
A total of 6 core biopsies were obtained from each primary cancer. Two core biopsy specimens were transferred immediately to liquid nitrogen and snap frozen at $-80°$ C. Each core biopsy measured approximately 1 cm x 1 mm. As these core biopsies were too small for micro dissection, we ascertained the tumor cellularity of the pretreatment core biopsies. In general, the core biopsies showed good tumor cellularity with median tumor cellularity of 75% (range 40-100%). Each core biopsy yielded 3-6 mg of total RNA, which is more than sufficient to generate approximately 20 mg of label cRNA needed for hybridization with the Affymetrix U95Av2 Genechip, using the manufacturer's standard protocols.

The clinical characteristics of the 24 patients enrolled in this phase II neoadjuvant study are included in Table 1. Before treatment, the median tumor size was 8 cm (range 4 to 30 cm). Prior to gene expression analysis, we defined sensitivity and resistance based on the percentage of residual disease after treatment. We first determined that the median residual disease after chemotherapy was 30%. We then arbitrarily defined sensitive tumors as those with 25% residual disease or less and resistant tumors as those with greater than 25% residual disease, as this cut-off divides the numbers of patients almost equally into two groups for statistical comparison. In addition, the presenting tumors were large in this study of locally advanced breast cancer, and tumor regressions of at least 75% following chemotherapy would almost certainly represent clinically responsive disease. Large tumor regressions following neoadjuvant chemotherapy have been shown to directly correlate with the probability of long-term survival [10].

Of these 24 patients, 11 were sensitive (46%) to docetaxel and 13 were resistant (54%). Of the sensitive tumors, 5 patients (5/11, 45%) had minimal residual disease (<10% residual tumor), while of the resistant tumors, 7 patients had residual tumors $\geq60\%$ (7/13, 58%), and 3 of these women (3/13, 23%) had residual tumors that were 100% or greater of baseline.

Each frozen core biopsy yielded 3 to 6μg of total RNA, which was more than sufficient to generate approximately 20μg of labeled cRNA needed for hybridization with the Affymetrix HgU95Av2 Gene Chip, using the manufacturer's standard protocol.

We compared the expression data in the sensitive and the resistant tumors to identify genes significantly differentially expressed between the two groups (Fig. 1). We first selected a subset of candidate genes by filtering on signal intensity to eliminate genes with uniformly low expression or genes whose expression did not vary significantly across the samples, retaining 1,628 genes. After log transformation, a t-test was used to select discriminatory genes. To evaluate the possibility of spurious results due to multiple comparisons, we performed a global permutation test, which evaluates the statistical probability of obtaining the observed number of differentially expressed genes (or more) by chance alone. T-tests with nominal P-values of 0.001, 0.01, and 0.05 selected respectively, 92, 300, and 551 genes as "differentially expressed". The probability that these numbers of genes would be selected by chance alone was estimated to be 0.0015, 0.001, and <0.001 respectively (Table 2). These results may be reviewed on www.ncbi.nlm.nih.gov/geo.

The 92 genes classed as most significantly "differentially expressed" at nominal P-value <0.001 are listed in the supplemental data (Fig. 1). These genes showed 4.2-2.6 fold decreases or 2.5-15.7 fold increases in expression in resistant *versus* sensitive tumors. Functional classes of these differentially expressed genes included stress/apoptosis (21%), cell adhesion/cytoskeleton (16%), protein transport (13%), signal transduction (12%), RNA transcription (10%), RNA splicing/transport (9%), cell cycle (7%), and protein translation (3%); the remainder (9%) had unknown functions.

Only 14 of the 92 genes were overexpressed in the resistant cluster with major categories including unknown function, protein translation, cell cycle, and RNA transcription, respectively. ß-tubulin isoforms were associated with docetaxel resistance.

Of the 78 genes overexpressed in docetaxel-sensitive tumors, major categories were stress/apoptosis, adhesion/cytoskeleton (none were overexpressed in resistant tumors), protein transport, signal transduction, and RNA splicing/transport. In sensitive tumors, genes involved in apoptosis (e.g., overexpression of BAX, UBE2M, UBCH10, CUL1), and DNA damage-related gene expression (e.g., overexpression of CSNK2B, DDB1, and ABL, and underexpression of PRKDC) appear to contribute to docetaxel sensitivity.

This current analysis will exclude some differential genes with low expression, some of which may be biologically interesting. For example, it has been proposed recently that spindle checkpoint dysfunction is an important cause of aneuploidy in human cancers. The serine-threonine kinase gene *AURORA-A* may constitute a mechanism of spindle checkpoint dysregulation, and its amplification has been shown to predict resistance to taxanes[24]. Indeed, we did observe differential expression was observed between sensitive and resistant tumors-overexpression of *AURORA-A* was approximately 1.4-fold higher in docetaxel-resistant *versus*

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sensitive tumors (Table 3). Nonetheless, this gene was not part of the 92-gene classifying list due to its overall low expression. This classifying list does not include all genes relevant to docetaxel sensitivity and resistance, but rather, identifies patterns of many genes that could be used as a predictive clinical test.

The feasibility of phenotype prediction with a linear classifier based on genes with a nominal P-value of 0.001 or better was tested with leave-one-out cross-validation. In this analysis, we began with all 1,628 filtered genes (see above) to overcome selection bias. [21,22] Each observation in turn was "left out", the remaining samples were used to select differentially expressed genes, and a compound covariate predictor was constructed and then used to classify the left-out sample. Ten of 11 sensitive tumors (specificity = 91%, exact binomial 95%CI 0.59-1.00) and 11 of 13 resistant tumors (sensitivity = 85%, 95% CI 0.55-0.98) were correctly classified, for an overall accuracy of 88% (95% CI = 68%-97%). Permutation testing indicates that such a high cross-validated classification accuracy is highly significant (P=0.008). The analogous predictor, constructed using 92 genes previously selected using all 24 samples, yielded identical classification success. Using this predictor, positive and negative predictive values for response to docetaxel were 92% and 83% respectively, and the area under the ordinary receiver operating characteristic (ROC) curve was 0.96 (Fig. 2).

Task 3.
To confirm measurement of RNA levels, expression values derived from normalized Affymetrix data were correlated with values from semi-quantitative RT-PCR (QRT-PCR) for fifteen genes (Table 4). Spearman rank correlations were positive for 13 genes and significantly positive for 6 of 15 genes.

Future directions

To validate this 92-gene predictive classifier, a subsequent cohort of 6 successive patients enrolled in this prospective clinical study was studied. In this small validation set, all 6 patients with sensitive tumors (residual disease less than 25%) were correctly classified by this classifier. We are future refining this gene predictor with additional samples collected from this completed neoadjuvant study.

Key Research Accomplishments

Four abstracts have been submitted and accepted for international meetings. Two were submitted to the San Antonio Breast Cancer Symposium in 2001 and 2002. This abstract was also submitted to the ASCO meeting in 2002 and 2003. This study was also selected for a preliminary presentation in the Era of Hope Meeting in Florida in 2002. A manuscript has been published in the prestigious medical journal, *The Lancet* in 2003. Another manuscript has been provisionally accepted in Journal of Clinical Oncology for 2004.

Reportable Outcomes

1. Genetic markers for response to neoadjuvant therapy: Array based gene expression profiling from serial biopsies. EC Wooten, **J Chang**, SG Hilsenbeck. 24th Annual San Antonio Breast Cancer Symposium, San Antonio, Texas (abstract 236), December 2001.

2. Gene expression profiles from breast cancer core biopsies predict therapy to response. EC Wooten, **J Chang**, SG Hilsenbeck. *Proceedings of the American Association for Cancer Research* 43, abstract 450, March 2002.

3. Gene expression profiles for doxytaxcil chemosensitivity. **J Chang**, EC Wooten and R Elledge. ASCO 28th Annual Meeting, abstract 1700, May 2002.

4. 4.**JC Chang**, EC Wooten, A Tsimelzon, SG Hilsenbeck, MC Gutierrez, R Elledge, S Mohsin, CK Osborne, GC Chamness, DC Allred, P O'Connell. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *The Lancet* 362:362-369, 2003.

5. **JC Chang,** EC Wooten, A Tsimelzon, SG Hilsenbeck, MC Gutierrez, R Elledge, S Mohsin, CKOsborne, G Chamness, DC Allred, MT Lewis, and P O'Connell. Patterns of acquired and *de* novo resistance, and incomplete response to docetaxel (Taxotere™) by gene expression profiling in breast cancer patients. Journal of Clinical Oncology (accepted) 2004.

Conclusions

We have preliminary data that gene expression patterns may predict response and resistance to chemotherapy. These patterns need to be better refined, and validated in subsequent studies. If patterns of gene expression exist that can help better select appropriate therapies, this would enable better selection of appropriate treatments for women with breast cancer.

Tables and Figures

Table 1. Clinical characteristics of patients enrolled in phase II study of neoadjuvant docetaxel. Pre and post indicates premenopausal and postmenopausal; ER, PR and HER-2 indicate estrogen receptor, progesterone receptor and HER-2/*neu* oncogene by immunohstochemistry, IMC denotes invasive mammary carcinoma, and IDC denotes invasive ductal carcinoma.

Subject	Age	Menopausal status	Ethnic Race	Presenting Tumor Size	Clinical Axillary Nodes	ER	PR	HER-2	Histologic Type
1	37	pre	Hispanic	10 x 10 cm	no	negative	negative	negative	IMC
2	55	post	Hispanic	10 x 8 cm	yes	negative	negative	positive	IDC
3	41	pre	Black	6 x 5 cm	yes	positive	positive	negative	IDC
4	43	pre	Black	15 x 13 cm	yes	positive	negative	negative	IMC
5	50	post	Black	20 x 23 cm	yes	negative	negative	negative	IDC
6	55	post	Black	11 x 11 cm	yes	positive	positive	negative	IDC
7	42	pre	Black	7 x 9 cm	yes	positive	positive	negative	IMC
8	63	post	Black	7 x 8 cm	yes	positive	positive	negative	IMC
9	50	post	Black	13 x 9 cm	no	positive	positive	negative	IDC
10	38	pre	Hispanic	8 x 8 cm	yes	positive	positive	negative	IMC
11	58	post	Hispanic	7 x 7 cm	yes	positive	positive	negative	IMC
12	62	post	Hispanic	4 x 4 cm	yes	positive	negative	negative	IDC
13	40	pre	Hispanic	5.5 x 4.5 cm	no	positive	positive	negative	IMC
14	36	pre	Black	6 x 6 cm	yes	positive	positive	negative	IDC
15	56	post	Black	5 x 5.5 cm	no	positive	negative	negative	IMC
16	38	pre	White	6 x 6 cm	yes	positive	negative	negative	IDC
17	54	post	White	5 x 6 cm	yes	positive	positive	positive	IDC
18	52	post	White	10 x 10 cm	no	positive	positive	negative	IDC
19	57	post	White	8 x 8cm	no	negative	negative	negative	IDC
20	52	post	Black	10 x 10 cm	no	negative	negative	negative	IDC
21	44	pre	Black	11 x 11 cm	no	negative	negative	negative	IDC
22	41	pre	Black	6 x 5 cm	yes	positive	positive	negative	IDC
23	38	pre	White	8 x 8 cm	yes	positive	positive	negative	IDC
24	54	post	Black	9 x 7 cm	no	positive	positive	negative	IDC

Table 2. Group comparison analysis, with different p-values (0.001, 0.01, 0.05). * Permutation p-value denotes the proportion of permutations in which the number of genes selected exceeds the observed number of genes selected.

P-value for gene selection	Number of differentially expressed genes	*Permutation p-value
0.001	92	0.0015
0.01	300	0.001
0.05	551	<0.001

Table 3. Differential expression of *AURORA-A*

Symbol	Probeset	Locuslink	Mean in Sensitive	Mean in Resistant	P-Value
STK6 (*Aurora A*)	34851_at	6790	506	695	0.046

Table 4. Correlation of Affymetrix expression data with SQ-RTPCR derived values. These correlations were positive for 13 genes and significantly positive for 6 of 15 genes.

Gene Symbol	Affymetrix Probe Set	N	Pearson Correlation		Spearman Rank Correlation	
			r	P value	r_{sp}	P Value
ACTB	32318_s_at	5	0.81	0.09	0.90	0.04
ATP6V0E	33875_at	5	0.28	0.65	0.10	0.87
BMI-1	1728_at	8	0.90	0.002	0.21	0.61
CALM3	1158_s_at	7	0.52	0.23	0.64	0.12
FUCA1	41814_at	6	0.77	0.07	0.94	0.00
GLRX	34311_at	8	0.74	0.03	0.50	0.21
IFITM1	676_g_at	5	0.74	0.15	0.70	0.19
LAMR1	256_s_at	8	0.69	0.06	0.85	0.01
LMNA	37378_r_at	5	-0.08	0.90	-0.40	0.50
MUC1	38783_at	8	0.84	0.01	0.71	0.05
MYO10	35362_at	8	0.15	0.72	0.05	0.91
PLOD	36184_at	4	-0.41	0.59	-0.80	0.20
PSMD5	32240_at	8	0.27	0.52	0.33	0.42
SERPINB5	863_g_at	8	0.75	0.03	0.81	0.01
SPARCL1	36627_at	6	0.92	0.01	1.00	0.00

Figure 1. Supervised hierarchical clustering, correlated with docetaxel response. Sensitive tumors (S) are defined as 25% residual disease or less (shown as blue bars), and resistant tumors (R) are defined as greater than 25% residual disease (shown as red bars). The expression levels are shown in red (expression levels above the median for the gene) and blue (levels below the median for the gene).
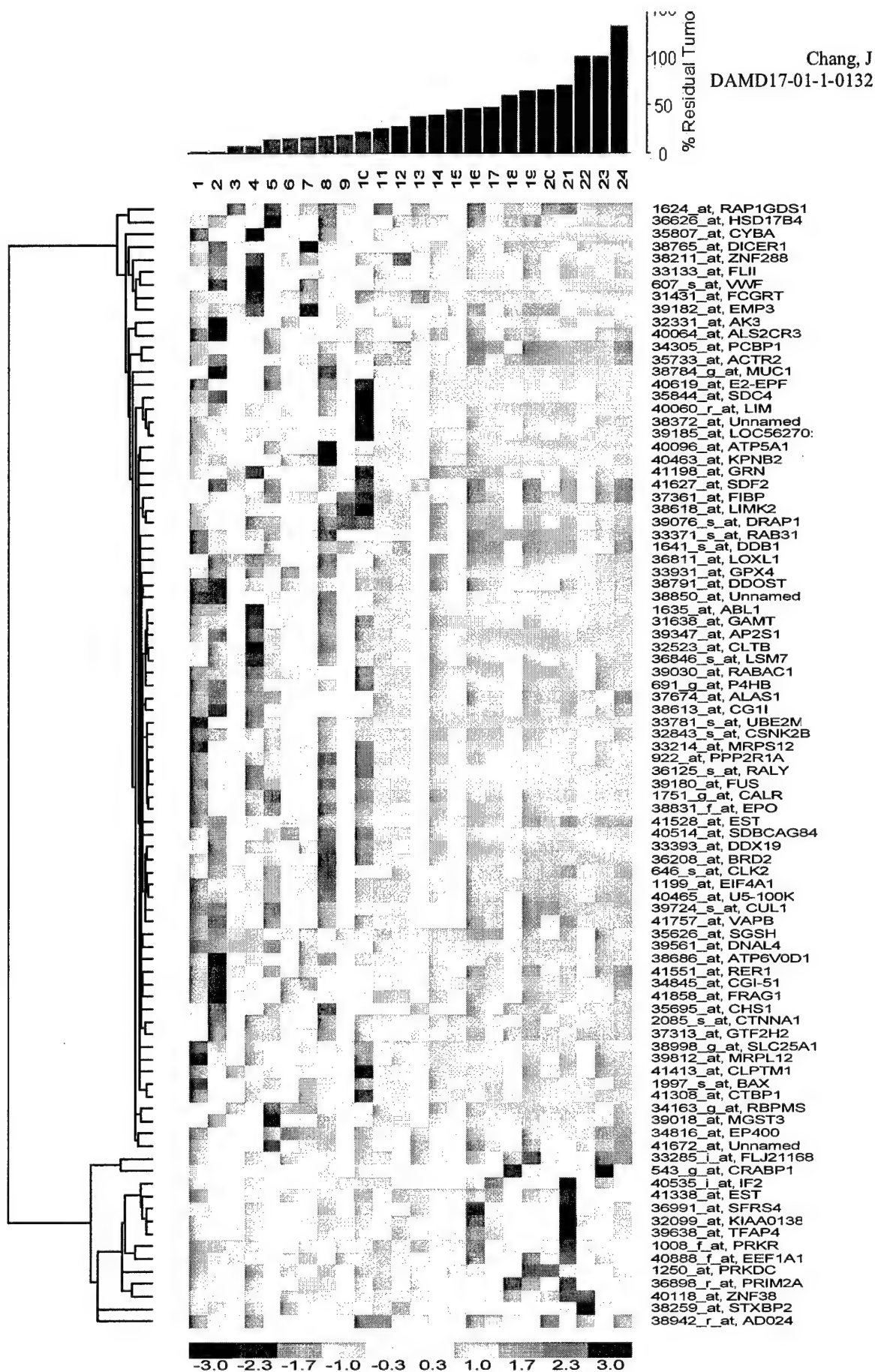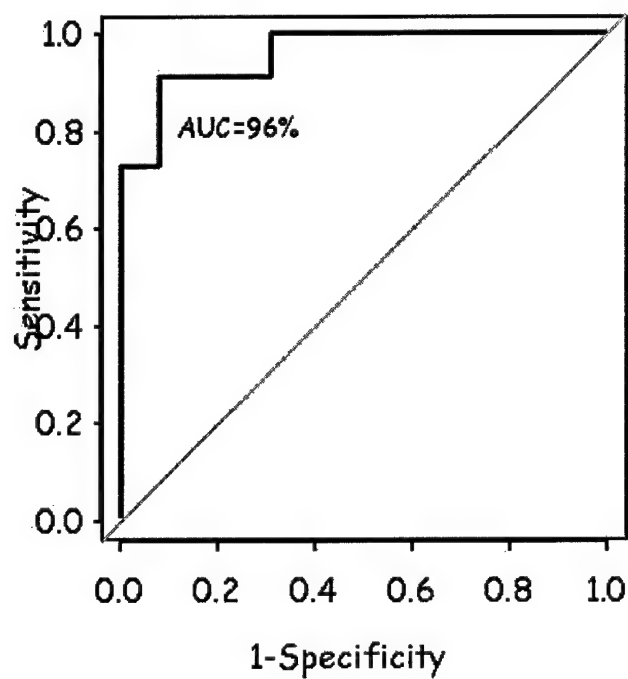
Figure 2. Receiver Operating Characteristic (ROC) Curve for docetaxel using the 92-gene classifier with positive and negative predictive values with 92% and 83% respectively, and the area under the curve is 0.96.

References

1. EBCTG. Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy: 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* 1992;**339**:1-15, 71-85.
2. The Early Breast Cancer Trialists' Collaborative Group E. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**351**(9114):1451-1467.
3. EBCTG. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998;**352**:930-942.
4. Hortobagyi GN. Docetaxel in breast cancer and a rationale for combination therapy. *Oncology* 1997;**11**(6):11-15.
5. Chan S, Friedrichs K, Noel D, et al. Prospective randomized trial of docetaxel versus doxorubicin in patients with metastatic breast cancer. The 303 Study Group. *J Clin Oncol* 1999;**17**(8):2341-54.
6. Nabholtz JM, Patterson A, Dirix L, Dewar J, Chap L, et al. A phase III trial comparing docetaxel (T), doxorubicin (A) and cyclophosphamide (C) (TAC) to (FAC) as first line chemotherapy for patients with metastatic breast cancer. *Proceedings of the American Society of Clinical Oncologists* 2001;**20**:22a.
7. Henderson IC BD, Demetri G, et al. Improved disease free survival and overall survival from the addition of sequential paclitaxel but not from escalation of doxorubicin in the adjuvant chemotherapy of patients with node-positive primary breast cancer. *Proc Am Soc Clin Onco* 1998;**17**:101.
8. Aapro MS. Adjuvant therapy of primary breast cancer: a review of key finding s from the 8th international conference, St. Gallen. *The Oncologist* 2001;**6**:376-385.
9. Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *Journal of Clinical Oncology* 1998;**16**(8):2672-2685.
10. Mamounas EP. Preoperative doxorubicin plus cyclophosphamide followed by preoperative or postoperative docetaxel. *Oncology* 1997;**11**(6 (Suppl 6)):37-40.
11. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;**270**(5235):467-470.
12. Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Research* 1998;**58**(22):5009-5013.
13. Lockhart DJ, Dong H, Byrne MC, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* 1996;**14**:1675-1680.
14. Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR, Elkahloun AG. In vivo gene expression profile analysis of human breast cancer progression. *Cancer Research* 1999;**59**(22):5656-5661.
15. Perou CM, Jeffrey SS, van de Run M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proceedings of the National Academy of Sciences of the United States of America* 1999;**96**:9212-9217.
16. van 't Veer LJ, Dai H, van De Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;**415**(6871):530-536.
17. Schadt EE, Li C, Ellis B, Wong WH. Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J Cell Biochem Suppl* 2001;**Suppl 37**:120-5.

18. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A* 2001;**98**(1):31-6.

19. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biology* 2001; **2**(8):research0032.1-0032.11.

20. Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. *J Comput Biol* 2002;**9**(3):505-11.

21. Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. *Proc Natl Acad Sci U S A* 2002;**99**(10):6562-6.

22. Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J Natl Cancer Inst* 2003;**95**(1):14-8.

23. McNeil BJ, Hanley JA. Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. *Med Decis Making* 1984;**4**(2):137-50.

24. Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* 2003;**3**(1):51-62.

25. Van Poznak C, Tan L, Panageas KS, et al. Assessment of molecular markers of clinical sensitivity to single-agent taxane therapy for metastatic breast cancer. *J Clin Oncol* 2002;**20**(9):2319-26.

26. Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* 1999;**17**(3):1061-70.

27. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;**347**(25):1999-2009.

28. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 2001;**98**(19):10869-74.

29. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000;**406**(6797):747-52.

30. Hansen RK, Parra I, Lemieux P, Oesterreich S, Hilsenbeck SG, Fuqua SA. Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. *Breast Cancer Res Treat* 1999;**56**(2):187-96.

31. Osborne CK, Yochmowitz MG, Knight WA, 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980;**46**(12 Suppl):2884-8.

COMMENTARY

Predictive cancer genomics—what do we need?

Neoadjuvant chemotherapy has been widely used before surgery for locally advanced breast cancer.[1,2] Good response to therapy facilitates breast-conserving surgery and has prognostic value, because it is associated with increased survival.[3,4] Similar preoperative treatment schedules are used in ovarian, bladder, and lung cancer, although the clinical benefit in these tumours has not yet been fully defined. Current clinical and pathological markers poorly predict response to chemotherapy, and neoadjuvant studies offer an ideal environment in which new molecular markers can be quickly correlated with clinical response. Markers discovered in this manner may also be of use for choosing therapy for adjuvant (postsurgical) treatment for micrometastatic disease.

To identify gene-expression signatures that might predict response to docetaxel in breast cancer, Jenny Chang and colleagues report in this issue of *The Lancet* the analysis of fresh material from 24 locally advanced cancers before chemotherapy in a phase II study. Samples were classed as sensitive or resistant to chemotherapy on the basis of the tumour residual volume at the end of treatment. Chang and colleagues profiled RNA expression in each sample with microarrays containing 12 625 gene probes, and then selected a subset of 1628 genes which had the highest variance across all the samples for further analysis. A classifier was then derived from the expression level of 91 genes with the most significant differential expression between sensitive and resistant cancers. This gene signature had a positive predictive value of 93% and a negative predictive value of 83% for response within the sample set from which it was derived.

Chang and colleagues' report is the first of many clinical trials that will use microarray technology to directly address which gene-expression patterns determine chemosensitivity in vivo. How should these analyses with this novel technology be considered and what conclusions can be drawn?[5] These questions are particularly pertinent when the biological significance of the gene-expression changes that correlate with a particular clinical outcome are not clear. First, it is essential that the data analysis takes account of the false-discovery rate that is inherent in microarray experiments when thousands of tests are done simultaneously on a small number of samples. These multiple tests require adjusted measures of significance; Chang's group used permutation testing to show that their findings were not expected by random chance. Second, classifiers derived from small series will be "overfitted" to the original dataset and may not have general applicability. As a minimum, the classifier needs to be applied to a subset of the sample that was not used for the original

derivation or preferably to an independent series. Chang and colleagues did a limited validation on an external set of six samples which were correctly classified, although the clinical details for the samples were not reported (see below). Third, it is important that the clinical studies are carefully designed with stringent criteria for assigning outcomes to samples.

In the Chang paper, the decision about what constituted a sensitive or resistant cancer was made at the end of the clinical study and was an arbitrary choice on the basis of the observed median residual volume of disease. However, the most important measure is pathological response, which is strongly correlated to survival.[3,4] Use of an arbitrary measure of tumour volume may not have any clinical relevance. Careful inspection of the cases shows that the partitioning method introduced other biases into the analysis. Sensitive and resistant cases differ in tumour size and histology (median perpendicular diameter 80 vs 36 cm, invasive ductal carcinoma 5/11 vs 11/13, invasive mammary carcinoma 6/11 vs 2/13). This difference is important because the size of the primary tumour mass is inversely correlated with response,[6] and so it is surprising that in this series the most responsive cancers had the largest median diameter. In addition it remains unclear whether response rates in ductal carcinoma differ from those in lobular cancers,[7] and Chang and colleagues do not break down their "invasive mammary carcinoma" classification into subtypes. Their classifier might therefore represent differences in size and histology rather than docetaxel sensitivity itself, which may explain why it does not include genes that have previously been associated with taxane resistance. The usefulness of the classifier can only be proven by testing it on larger independent datasets.

Perhaps the strongest argument for reporting exploratory clinical studies, such as that of Chang and colleagues, is their potential for reuse in other analyses, which can then aggregate multiple datasets. As with all gene-expression reports in *The Lancet*,[8] the raw array data will be available for reanalysis from public microarray repositories.[9] However, to make best use of these datasets, clinical details must also be fully reported and be electronically accessible. At present the ontological descriptions and database structures to facilitate this process are being studied.[10] In the UK, the National Cancer Research Institute and the National Translational Cancer Research Network are evaluating new clinical bioinformatics initiatives, partly in collaboration with the US National Cancer Institute Centre for Bioinformatics. The use of controlled vocabularies and concept indexes in clinical databases will be an important first step. Data from clinical studies

and molecular profiling need to be tightly coupled and widely accessible if the promise of predictive cancer genomics is to be achieved.

We have no conflicts of interest to declare.

*James D Brenton, Carlos Caldas

Cancer Genomics Program, Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge CB2 2XZ, UK
(e-mail: jdb1003@cam.ac.uk)

1 Bonadonna G, Veronesi U, Brambilla C, et al. Primary chemotherapy to avoid mastectomy in tumors with diameters of three centimeters or more. J Natl Cancer Inst 1990; 82: 1539–45.

2 Smith IE, Jones AL, O'Brien ME, McKinna JA, Sacks N, Baum M. Primary medical (neo-adjuvant) chemotherapy for operable breast cancer. Eur J Cancer 1993; 29A: 1796–99.

3 Kuerer HM, Newman LA, Smith TL, et al. Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. J Clin Oncol 1999; 17: 460–69.

4 Choller P, Amat S, Cure H, et al. Prognostic significance of a complete pathological response after induction chemotherapy in operable breast cancer. Br J Cancer 2002; 86: 1041–66.

5 Petricoin EF 3rd, Hackett JL, Lesko LJ, et al. Medical applications of microarray technologies: a regulatory science perspective. Nat Genet 2002; 32: 474–79.

6 Faneyte IF, Schrama JG, Peterse JL, Remijnse PL, Rodenhuis S, van de Vijver MJ. Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome. Br J Cancer 2003; 88: 406–12.

7 Cocquyt VF, Blondeel PN, Depypere HT, et al. Different responses to preoperative chemotherapy for invasive lobular and invasive ductal breast carcinoma. Eur J Surg Oncol 2003; 29: 361–67.

8 Ball CA, Sherlock G, Parkinson H, on behalf of Microarray Gene Expression Data (MGED). A guide to microarray experiments—an open letter to the scientific journals. Lancet 2002; 360: 1019.

9 NCBI. Gene expression omnibus. http://www.ncbi.nlm.nih.gov/geo (accessed July 29, 2003).

10 National Cancer Institute. caCORE: The NCICB Cancer Informatics Infrastructure Backbone. http://ncicb.nci.nih.gov/core (accessed July 29, 2003).

(Almost) three cheers for UK genetics White Paper

£50 million over 3 years is a drop in the ocean compared with the annual UK National Health Service (NHS) budget of £54 billion. The importance of the UK Government's White Paper on genetics,[1] unveiled by Health Secretary John Reid on June 24, 2003, lies not so much in its financial promises as in the signal it sends: that the Government recognises the importance of genetics for the health service and is making a strategic commitment to its development.

No one knows how soon the promises made for genetics will become a reality or how deep the eventual impact will be.[2] Recognising this uncertainty, the White Paper attempts to steer a line between enthusiasm for the benefits genetic medicine may bring[3,4] and the danger of raising unrealistic expectations.[5] By and large the White Paper succeeds, though the antigenetics lobby groups have criticised some of the paper's scenarios. It is too early to say, as the report suggests: whether patients who have heart attacks would really be more motivated to take preventive action if they know they are at increased genetic risk for coronary heart disease; whether future genetic tests will add an appreciable degree of precision to current risk estimates based on biochemical or lifestyle factors; or, indeed, whether existing interventions will be of greater or lesser effect in genetically predisposed individuals.

Some models suggest that combinations of low-penetrance genes may have a major role in determining individual risk of common disorders.[6] However, there is a long way to go in validating the many proposed associations between genotype and disease risk, and in rigorous assessment of genetic tests to determine not only their analytical and clinical validity, but also their clinical usefulness and their ethical, legal, and social impact.[7] The White Paper makes passing reference to the need for an evidence-based approach to genetic medicine, but could usefully have made issues surrounding assessment and regulation of genetic tests a more prominent plank of its proposals.

That said, there is much to applaud in the choice of priorities set out in the White Paper. In anticipation of growing numbers of genetic tests coming into service, genetics laboratories are in line to receive a boost in funding of £22·5 million. But there is no such thing as a free sequencing machine: the White Paper makes it clear that this funding is "in return for innovative plans for modernisation developed to meet local needs". There are broad hints that the Government foresees a time, perhaps not very far in the future, when genetic testing may be integrated into wider pathology services, with economies of scale from geographical re-distribution and centralisation of testing, and perhaps greater involvement of private sector partners.[8] Genetics laboratories have given sterling service in bringing tests for single-gene disease into the NHS but the time is now right to begin thinking about new service models that will serve the needs not only of those with genetic disorders but also all groups of patients.

Also to be welcomed is a commitment to improve awareness of genetics in mainstream services such as those for cancer and heart disease, in which much can now be done to improve the recognition and management of familial subsets of these diseases. Familial hypercholesterolaemia, familial cancer, and maturity onset diabetes of the young are singled out for special attention in the White Paper, but it should not be forgotten that conditions such as long QT syndrome, cardiomyopathies, or polycystic kidney disease are also strong candidates for a family-based approach, nor that a more directed use of family history as a guide to disease risk might be of use in primary care.[9]

Such developments must go hand in hand with a greater awareness and understanding of genetics by all health professionals. Anyone who has run a genetics education event for the health service will know that the current NHS workforce is not falling over itself to improve its genetic literacy. This unenthusiasm is not surprising, given the heavy demands of targets and waiting lists and the long time-frame before genetics will make a substantial impact on day-to-day practice. The results of a review of genetic education in health professionals make it clear that, to have any hope of success, programmes must be developed in partnership with each professional group and rooted in their clinical experience.[10] Starry-eyed pronouncements about the wonders of the Human Genome Project reach only the already converted. In this context, it is encouraging that the NHS Genetics Education and Development Centre, announced in the White Paper, will "act as a catalyst to help drive and coordinate activity", rather than dictating what different professionals need to know. It is less clear that the Government is prepared to make available sufficient resources for genetic education of health professionals. If real progress is to be made,

Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer

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Eric C Wooten

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Peter O'Connell

Mechanisms of disease

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Summary

Background Systemic chemotherapy for operable breast cancer substantially decreases the risk of death. Patients often have de novo resistance or incomplete response to docetaxel, one of the most active agents in this disease. We postulated that gene expression profiles of the primary breast cancer can predict the response to docetaxel.

Methods We took core biopsy samples from primary breast tumours in 24 patients before treatment and then assessed tumour response to neoadjuvant docetaxel (four cycles, 100 mg/m² daily for 3 weeks) by cDNA analysis of RNA extracted from biopsy samples using HgU95-Av2 GeneChip.

Findings From the core biopsy samples, we extracted sufficient total RNA (3–6 μg) for cDNA array analysis using HgU95-Av2 GeneChip. Differential patterns of expression of 92 genes correlated with docetaxel response ($p=0.001$). Sensitive tumours had higher expression of genes involved in cell cycle, cytoskeleton, adhesion, protein transport, protein modification, transcription, and stress or apoptosis; whereas resistant tumours showed increased expression of some transcriptional and signal transduction genes. In leave-one-out cross-validation analysis, ten of 11 sensitive tumours (90% specificity) and 11 of 13 resistant tumours (85% sensitivity) were correctly classified, with an accuracy of 88%. This 92-gene predictor had positive and negative predictive values of 92% and 83%, respectively. Correlation between RNA expression measured by the arrays and semiquantitative RT-PCR was also ascertained, and our results were validated in an independent set of six patients.

Interpretation If validated, these molecular profiles could allow development of a clinical test for docetaxel sensitivity, thus reducing unnecessary treatment for women with breast cancer.

Breast Center and the Departments of Medicine, Pathology, and Molecular and Cellular Biology, Baylor College of Medicine, and the Methodist Hospital, Houston, TX USA (J C Chang MD, EC Wooten PhD, A Tsimelzon PhD, Prof S G Hilsenbeck PhD, M C Gutierez MD, R Elledge MD, S Mohsin MD, Prof C K Osborne MD, Prof G C Chamness PhD, Prof D C Allred MD, Prof P O'Connell PhD)

Correspondence to: Dr Jenny Chang, Breast Center, Baylor College of Medicine, One Baylor Plaza, BCM 600, Houston, TX 77030, USA (e-mail: jcchang@breastcenter.tmc.edu)

Introduction

Adjuvant systemic treatment after surgery for breast cancer is the most crucial factor in reducing mortality—both chemotherapy and hormonal treatment reduce the risk of death in such patients.[1-4] However, although oestrogen-receptor status is predictive of response to hormonal treatments, there are no clinically useful predictive markers of a patient's response to chemotherapy. Therefore, all patients who are eligible for chemotherapy receive the same treatment, even though de novo drug resistance will result in treatment failures in many. The taxanes, docetaxel and paclitaxel, are a new class of antimicrotubule agent that are more effective than older drugs such as anthracyclines,[5-7] although results of clinical trials with taxanes and anthracyclines in combination show that only a small subset of patients benefit from the addition of taxanes.[8,9] There are no methods to distinguish between patients who are likely to respond to taxanes and those who are not. In view of the accepted practice of giving adjuvant treatment to most patients, even if the average expected benefit is low, the a priori selection of appropriate patients most likely to benefit from adjuvant treatment with taxanes would be a great advance in the clinical management of breast cancer.[8,9] A major impediment in the study of predictors of effectiveness of adjuvant treatment is the absence of surrogate markers for survival and, consequently, large numbers of patients and long-term follow-up are needed.

We aimed to identify gene expression patterns in primary breast-cancer specimens that might predict response to taxanes. Neoadjuvant chemotherapy (ie, treatment before primary surgery) allows for sampling of the primary tumour for gene expression analysis, and for direct assessment of response to chemotherapy by monitoring changes in tumour size during the first few months of treatment.[10,11] Clinical response of the tumour to neoadjuvant chemotherapy is a valid surrogate marker of survival: patients whose tumours regress substantially after neoadjuvant chemotherapy have better outcome than do those with modest response or clinically obvious disease that is resistant to chemotherapy.[10,11] With the advent of high-throughput quantification of gene expression, simultaneous assessment of thousands of genes is now possible, which allows identification of expression patterns in different breast cancers that might correlate with, and thereby predict, excellent clinical response to treatment.[12-16] These profiles have potential to explain the genetic heterogeneity of breast cancer and allow treatment strategies to be planned in accordance with their probability of success in individual patients. Hence, neoadjuvant chemotherapy provides an ideal platform from which to discover predictive markers of chemotherapy response. In our study, we took core needle biopsy samples of the primary breast cancer for gene expression profiling before patients received neoadjuvant docetaxel. We aimed first, to show that sufficient RNA

could be obtained from core biopsy samples to assess gene expression; second, to identify groups of genes that could be used to distinguish primary breast cancers that are responsive or resistant to docetaxel chemotherapy; and third, to identify gene pathways that could be important in the mechanism of resistance to docetaxel.

Methods

Patients

From September, 1999, to June, 2001, patients with locally advanced breast cancer (ie, primary cancers >4 cm, or clinically evident axillary metastases) were considered for a phase II study with neoadjuvant docetaxel. Inclusion criteria were (1) age greater than 18 years and a diagnosis of breast cancer confirmed by analysis of a core needle biopsy sample, (2) premenopausal status accompanied by appropriate contraception, (3) adequate performance status, and (4) adequate liver and kidney function tests (all within 1·5 times the institution's upper limit of normal). Patients were excluded if they had severe underlying chronic illness or disease, or were taking other chemotherapeutic drugs while on study.

This study (protocol H8448) was approved by the institutional review board of Baylor College of Medicine, Houston, TX, USA. Patients gave written informed consent.

Clinical procedures

We recorded clinical staging and size of primary tumour at the start of treatment, at every cycle, and after completion of four cycles of chemotherapy. Tumour size (product of the two largest perpendicular diameters) measured before and after four cycles of neoadjuvant chemotherapy was used to calculate the percentage of residual disease. The median residual disease was then calculated, and this degree of response was used to divide the cancers into two roughly equal groups—sensitive and resistant tumours—before we did gene expression analysis.

Before docetaxel was given, we did core biopsies of the primary cancers. To obtain sufficient tissue, we did about six core biopsies from every patient using an MC1410 MaxCore biopsy instrument (Bard, Covington, GA, USA). Samples were taken after patients had been given local anesthesia with the same entry point, but reorienting the needle. Two to three core biopsy specimens were immediately transferred for snap freezing at –80°C for cDNA array analysis. The remaining specimens were fixed in formalin for diagnostic analysis and possible immunohistochemical analysis.

Four cycles of docetaxel were given at 100 mg/m^2 every 3 weeks, and we assessed clinical response after the fourth cycle, at 12 weeks. As part of standard care, patients were continued on neoadjuvant chemotherapy through the full four cycles, unless there was clear documentation of progressive disease, which we defined as an increase in tumour size of more than 25%. After the course of neoadjuvant docetaxel was complete, primary surgery was done and standard adjuvant treatment was given.

RNA extraction and amplification

We isolated total RNA from the frozen core biopsy specimens in accordance with protocols recommended by Affymetrix (Santa Clara, CA, USA) for GeneChip experiments. Total RNA was isolated with Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Samples were subsequently passed over a Qiagen RNeasy column (Qiagen, Valencia, CA) for removal of small fragments that affect RT-reaction and hybridisation quality (ECW, unpublished data). Each core biopsy yielded 3–6 µg of total RNA. After RNA recovery, double-stranded cDNA was then synthesised by a chimeric oligonucleotide with an oligo-dT and a T7 RNA polymerase promoter at a concentration of 100 pmol/µL.

We did reverse transcription in accordance with protocols recommended by Affymetrix using commercially available buffers and proteins (Invitrogen Corporation). Biotin labelling and about 250-fold linear amplification followed phenol-chloroform clean up of the reverse-transcription reaction product and was done by in-vitro transcription (Enzo Biochem, New York, NY, USA) over a reaction time of 8 h. From each biopsy specimen, we hybridised 15 µg of labelled cRNA onto the HgU95-Av2 GeneChip using recommended procedures for pre-hybridisation, hybridisation, washing, and staining with streptavidin-phycoerythrin (SA-PE). Antibody amplification was done with a biotin-linked antibody to streptavidin (Vector Laboratories, Burlingame, CA) with a goat-IgG blocking antibody (Sigma, St Louis, MO, USA). A second application of the SA-PE dye was used after additional wash steps had been done. After automated staining and wash protocols (Affymetrix protocol EukGE-2v4), the arrays were scanned by the Affymetrix GeneChip scanner (Agilent, Palo Alto, CA) and quantitated with Micoarray suite version 5.0 (Affymetrix). The HgU95-Av2 GeneChip consists of about 12 625 probe sets, each containing about 16 perfect match and corresponding mismatch 25mer oligonucleotide probes representing sequences (genes), most of which have been characterised in terms of function or disease association. The raw, unnormalised probe level data were then analysed by dChip (http://dchip.org) for final normalisation and modelling. Median intensity was used for the normalisation of the 24 arrays and the perfect match/mismatch (PM/MM) modelling algorithm was used.

Semiquantitative RT-PCR

We did semi-quantitative RT-PCR (sqRT-PCR) measurement of gene expression levels using the same amplified cRNA hybridised to the GeneChip. 20 genes were selected for analysis on the basis of their high variation in expression. Primers were designed for these loci with the sequences freely available from the Entrez Nucleotide database[17] and the Primer3 algorithm for primer design. Product sizes were kept short (<150 bp) to allow the maximum ability to work under varying conditions relative to cRNA quality. Primers were optimised with a reverse-transcribed mixture of six samples. 15 duplicate reactions were prepared and samples were taken at alternating cycle numbers between 15 and 33 to ensure that the sqRT-PCR reaction products were in a linear range of accumulation. These samples were then arranged in ascending order, diluted with 10 µL loading buffer, and 3 µL of each sample was loaded onto 6% denaturing acrylamide gels. Electrophoresis at 60 W was done for 2 h, or until sufficient separation of the xylene cyanol and bromophenol blue dyes
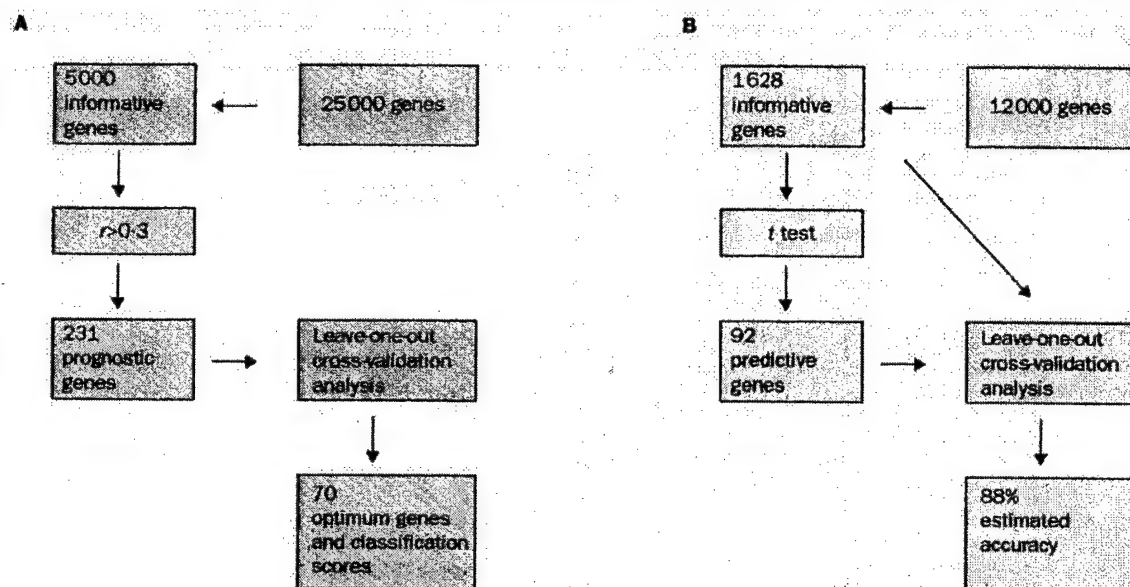
Figure 1: Two methods of statistical analysis
A: the prognostic analysis used by van't Veer and colleagues[18] used oligonucleotide microarrays with 25 000 genes, from which 5000 variably expressed genes were selected by filtering. Of these, 231 genes were significantly associated with prognostic outcome (r>0·3). These 231 genes were then rank-ordered on the basis of the magnitude of the correlation coefficient and selected in groups of five to construct the smallest optimum classifier. Leave-one-out analysis was then done with 231 genes that were correlated with outcome to select a classification set of 70 genes. B: statistical analysis methods used in this study: a subset of 1628 genes was selected by filtering on signal intensity to eliminate genes with uniformly low expression or genes whose expression did not vary significantly across the samples.

was achieved. Gels were then fixed, removed from the rear plate, transferred to filter paper, and dried. We first assessed these dry gels using autoradiography (about 8 h exposure, no intensification), and analysable gels were then exposed to phosphorimaging screens. Primers that failed to produce a single clear band were used again with different annealing temperatures until a single band was produced.

15 of the 20 primers chosen proved suitable to use and gave clean, single bands for analysis. The remaining five failed to optimise properly and were not included in any further analysis. Although high-cycle samples inevitably achieved pixel-saturation, care was taken to keep exposure times to a minimum, so as to keep intensity within the informative range on most cycle-totals within each set. To determine the linear range of the 15 primers, we analysed their absolute intensities using Microsoft Excel graphing functions. We then did phosphorimager quantification analysis (Bio-Rad Laboratories, Hercules, CA), and RT-PCR product band intensities were quantitatively compared with normalised, model-based estimates of expression from the GeneChip data.

Statistical analysis
The analytical approach used in this study (figure 1) was similar to the successful methods described previously.[18] After scanning and low-level quantification using Microarray Suite (Affymetrix), we used DNA-Chip analyser dChip version 1.2 to adjust arrays to a common baseline[19] and estimated expression using Li and colleagues' PM-MM model.[20,21] We eliminated genes that were not present in at least 30% of samples, and exported expression data for the remaining 6849 genes to BRB Arraytools version 2.1c[22] for more filtering and analysis. After transforming all data by taking logarithms, we ranked genes by variability over all 24 samples, and we retained the 1628 genes that were significantly more variable than the median variance.

We selected differentially expressed genes from the filtered gene list using the two-sample t test, and then used a global permutation test as an overall, multiple comparison-free test of whether the number of differentially expressed genes exceeded that which might arise by chance. In this test, the observed number of significantly differentially expressed genes was compared with the distribution of numbers of differentially expressed genes generated by repeatedly permutating the labels of the samples and recalculating the t test at the specified level of significance.

Next, we developed a classifier to predict response. With a list of discriminatory genes and their associated t values, we used the compound covariate predictor method of Radmacher and colleagues[23] to construct a linear classifier. RESUBSTITUTION ESTIMATES of classification success, in which the classifier is applied to the same samples used to create it, are invariably biased (ie, they are overly optimistic).[24,25] Therefore, we used an external cross-validation procedure to generate a less biased estimate of classification success. Starting with 1628 genes that had significant variation in expression, and which were filtered without any respect to class membership, the entire gene selection and classifier construction process was repeated in a leave-one-out cross-validation to estimate classifier performance. Finally, to assess whether the degree of successful classification we noted could have arisen by chance, the entire cross-validation procedure was repeated 2000 times, permutating the sample labels every time. The observed cross-validated classification success rate was then compared with the distribution of classification success in the permutation analysis. Cross-validated performance was summarised by observed sensitivity and specificity, and associated exact binomial confidence intervals. Resubstitution classifier values were also used to generate a receiver operating characteristic curve (ROC curve) and to estimate the area under the curve.

The classifier was partly validated with an independent set of six patients treated in the same clinical trial as those

	Age (years)	Menopausal status	Ethnic origin	Bidimensional tumour size (cm)	Clinical axillary nodes	Oostrogen-receptor status	Progesterone-receptor status	HER-2	Tumour type
Patient									
1	37	Premenopausal	Hispanic	10×10	No	–	–	–	IMC
2	55	Postmenopausal	Hispanic	10×8	Yes	–	–	+	IDC
3	41	Premenopausal	Black	6×5	Yes	+	+	–	IDC
4	43	Premenopausal	Black	15×13	Yes	+	–	–	IMC
5	50	Postmenopausal	Black	20×23	Yes	–	–	–	IDC
6	55	Postmenopausal	Black	11×11	Yes	+	+	–	IDC
7	42	Premenopausal	Black	7×9	Yes	+	+	–	IMC
8	63	Postmenopausal	Black	7×8	Yes	+	+	–	IMC
9	50	Postmenopausal	Black	13×9	No	+	+	–	IDC
10	38	Premenopausal	Hispanic	8×8	Yes	+	+	–	IMC
11	58	Postmenopausal	Hispanic	7×7	Yes	+	+	–	IMC
12	62	Postmenopausal	Hispanic	4×4	Yes	+	–	–	IDC
13	40	Premenopausal	Hispanic	5·5×4·5	No	+	+	–	IMC
14	36	Premenopausal	Black	6×6	Yes	+	+	–	IDC
15	56	Postmenopausal	Black	5×5·5	No	+	–	–	IMC
16	38	Premenopausal	White	6×6	Yes	+	–	–	IDC
17	54	Postmenopausal	White	5×6	Yes	+	+	+	IDC
18	52	Postmenopausal	White	10×10	No	+	+	–	IDC
19	57	Postmenopausal	White	8×8	No	–	–	–	IDC
20	52	Postmenopausal	Black	10×10	No	–	–	–	IDC
21	44	Premenopausal	Black	11×11	No	–	–	–	IDC
22	41	Premenopausal	Black	6×5	Yes	+	+	–	IDC
23	38	Premenopausal	White	8×8	Yes	+	+	–	IDC
24	54	Postmenopausal	Black	9×7	No	+	+	–	IDC

HER-2=HER-2/neu oncogene detected by immunohistochemical analysis. –=negative. +=positive. IMC=invasive mammary carcinoma. IDC=invasive ductal carcinoma.

Table 1: **Characteristics of patients in the training set**

in the training set. RNA was obtained from pretreatment biopsy samples and hybridised to HgU95-Av2 GeneChips exactly as described for the training sample. Probe level data were adjusted to the same baseline array as the training set, and gene expression values were calculated with previously estimated probe sensitivity values derived from the training sample. The 92-gene classifier was then applied to predict response in every new sample.

Role of the funding source
The study sponsors did not contribute to the study design, or collection, analysis, or interpretation of data. The manuscript was reviewed with only minor editorial changes by one of the study's sponsors, Aventis Pharmaceutical.

Results
Assessment of clinical response
We included 24 patients, and their clinical characteristics are shown in table 1. Unidimensional median tumour size before treatment was 8 cm (range 4–23 cm). Before doing gene expression analysis, we defined tumour sensitivity and resistance on the basis of the percentage of residual disease after treatment. We first determined that the median residual disease after chemotherapy was 30%. We then arbitrarily defined sensitive tumours as those that had 25% or less residual disease, and resistant tumours as those with more than 25% residual disease, since this cutoff divides the patients into two almost equally sized groups for statistical comparison. In this study of locally advanced breast cancer, tumours were large and a regression of at least 75% after chemotherapy would almost certainly represent a clinically important response. Of these 24 patients, 11 (46%) were sensitive to docetaxel and 13 (54%) were resistant. Of the sensitive tumours, five patients (45%) had minimal residual disease (<10% residual tumour), whereas of the resistant tumours, seven (58%) had residual tumour mass of 60% or greater, and three (23%) of these residual tumours were 100% or greater of baseline.

Selection of discriminatory genes
To select discriminatory genes, we compared expression data in the sensitive and the resistant tumours (figure 2). First, we selected a subset of candidate genes by filtering on signal intensity to eliminate genes with uniformly low expression or genes whose expression did not vary significantly across the samples, retaining 1628 genes. After log transformation, a t test was used to select discriminatory genes. t tests with nominal p values of 0·001, 0·01, and 0·05 selected 92, 300, and 551 genes, respectively, for which expression differed in sensitive and resistant groups—ie, differentially expressed. The probability that these numbers of genes would be selected by chance alone was estimated to be 0·0015, 0·001, and less than 0·001 respectively (table 2). These results can be reviewed with data at the gene expression omnibus.[16]

Functional classification of discriminatory genes
The 92 genes classed as most significantly "differentially expressed" at p=0·001 are listed in the webtable (http://image.thelancet.com/extras/01art11086webtable.pdf) (figure 2). These genes showed 2·6–4·2-fold decreases or 2·5–15·7-fold increases in expression in resistant compared with sensitive tumours. Functional classes of these differentially expressed genes included stress or APOPTOSIS (21%), cell adhesion or cytoskeleton (16%), protein transport (13%), signal transduction (12%), RNA transcription (10%), RNA splicing or transport (9%), cell cycle (7%), and protein translation (3%); the remainder (9%) have unknown functions. 14 of these 92 genes were overexpressed in the treatment-resistant cluster with major categories including unknown function, protein

	p value for gene selection		
	0·001	0·01	0·05
Number of differentially expressed genes	92	300	551
Permutation p*	0·0015	0·001	0·001

*The proportion of permutations in which the number of genes selected exceeds the observed number of genes.

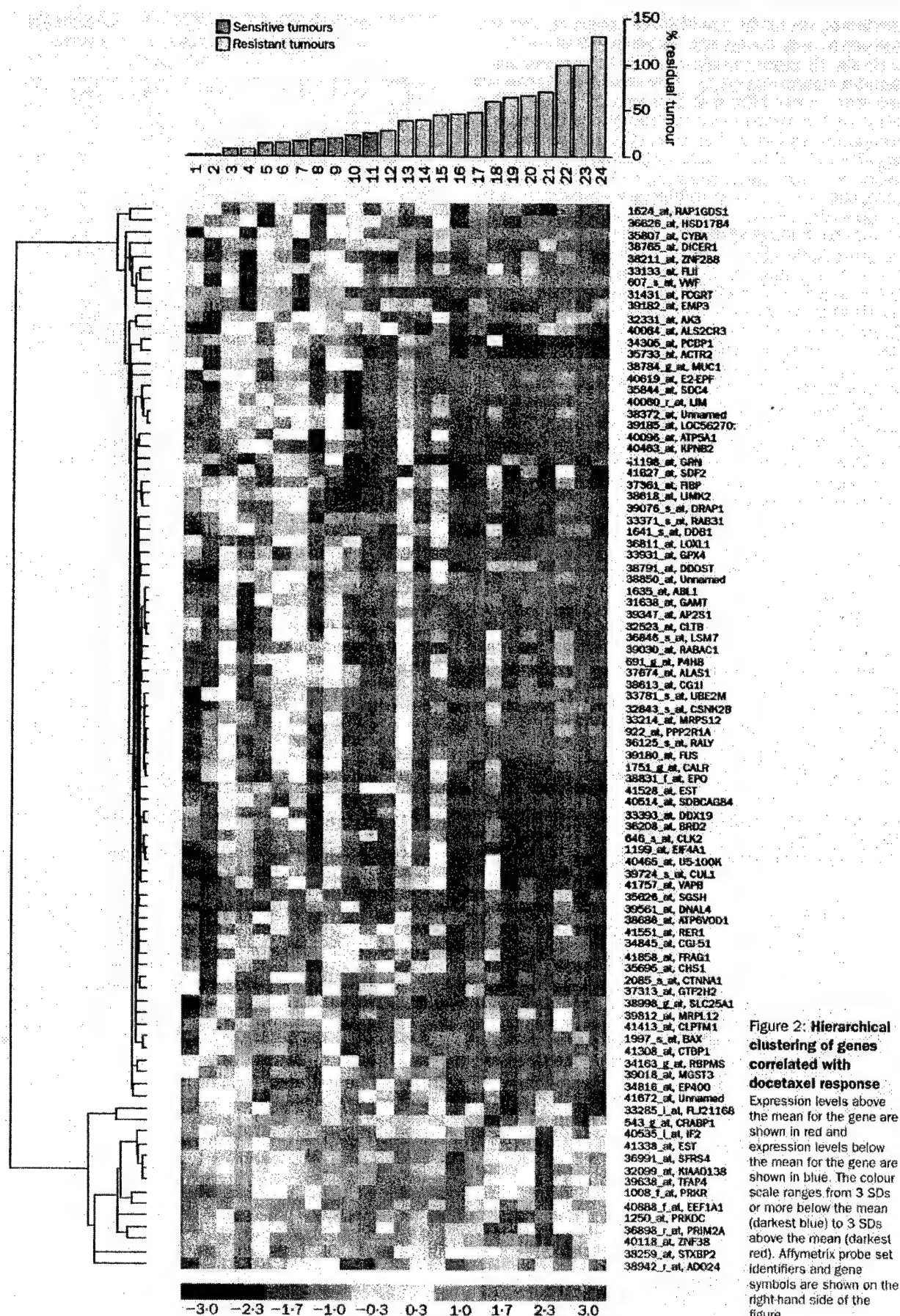Table 2: **Group comparison analysis, with different nominal p-values**

Figure 2: **Hierarchical clustering of genes correlated with docetaxel response**
Expression levels above the mean for the gene are shown in red and expression levels below the mean for the gene are shown in blue. The colour scale ranges from 3 SDs or more below the mean (darkest blue) to 3 SDs above the mean (darkest red). Affymetrix probe set identifiers and gene symbols are shown on the right-hand side of the figure.

translation, cell cycle, and RNA transcription. Tubulin isoforms were associated with docetaxel resistance.

Of the 78 genes overexpressed in docetaxel-sensitive tumours, major categories were stress or apoptosis, adhesion or cytoskeleton (no genes with this function were overexpressed in resistant tumours), protein transport, signal transduction, and RNA splicing or transport. In sensitive tumours, genes involved in apoptosis (eg, overexpression of *BAX*, *UBE2M*, *UBCH10*, *CUL1*), and DNA damage-related gene expression (eg, overexpression of CSNK2B, DDB1, and *ABL1*, and underexpression of *PRKDC*) seem to contribute to docetaxel sensitivity.

Leave-one-out cross-validation

In this cross-validation analysis, we began with all 1628 filtered genes to avoid selection bias.[24,25] Every observation in turn was left out and the remaining samples were used to select differentially expressed genes; we then constructed a compound covariate predictor to classify the left-out sample. Ten of 11 sensitive tumours (91% specificity, [95%CI 0·59–1·00]) and 11 of 13 resistant tumours (85% sensitivity [0·55–0·98]) were correctly classified, for an overall accuracy of 88% (68–97%). Results of permutation testing showed that such a high cross-validated classification accuracy is significant (p=0·008). The analogous predictor, constructed with 92 genes selected with use of all 24 samples, yielded identical classification success. With this predictor, positive and negative predictive values for response to docetaxel were 92% and 83%, respectively, and the area under the ordinary receiver operating characteristic (ROC) curve was 0·96 (figure 3).

Confirmation of expression measurements

To confirm measurement of RNA concentrations, expression values derived from adjusted Affymetrix data were correlated with values from sqRT-PCR for 15 variably expressed genes (table 3). Spearman rank correlations were positive for 13 genes and significantly positive for six of 15 genes.

Validation in an independent cohort

The six additional patients enrolled in this prospective clinical study were studied to partly validate the 92-gene
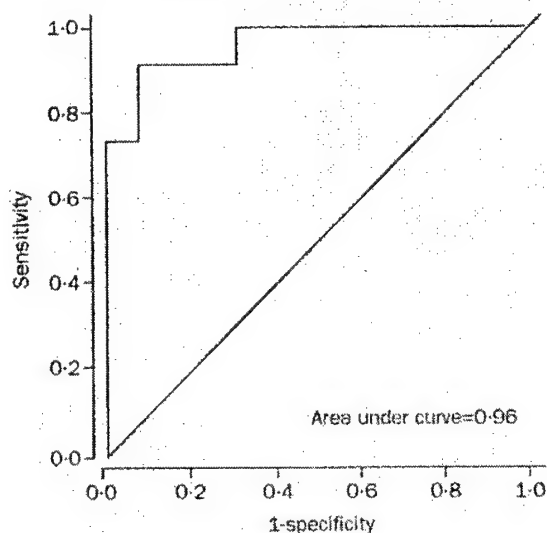

Figure 3: **Receiver operating characteristic (ROC) curve for predicting response to docetaxel**

	Affymetrix probe set	Number	Pearson correlation		Spearman rank correlation	
			r	p	r_s	p
ACTB	32318_s_at	5	0·81	0·09	0·90	0·04
ATP6VOE	33875_at	5	0·28	0·65	0·10	0·87
BMI-1	1728_at	8	0·90	0·002	0·21	0·61
CALM3	1158_s_at	7	0·52	0·23	0·64	0·12
FUCA1	41814_at	6	0·77	0·07	0·94	0·00
GLRX	34311_at	8	0·74	0·03	0·50	0·21
IFITM1	676_g_at	5	0·74	0·15	0·70	0·19
LAMR1	256_s_at	8	0·69	0·06	0·85	0·01
LMNA	37378_r_at	5	−0·08	0·90	−0·40	0·50
MUC1	38783_at	8	0·84	0·01	0·71	0·05
MYO10	35362_at	8	0·15	0·72	0·05	0·91
PLOD	36184_at	4	−0·41	0·59	−0·80	0·20
PSMD5	32240_at	8	0·27	0·52	0·33	0·42
SERPINB5	863_g_at	8	0·75	0·03	0·81	0·01
SPARCL1	36627_at	6	0·92	0·01	1·00	0·00

Correlations positive for 13 genes and significantly positive for 6 of 15 genes

Table 3: **Correlation of Affymetrix expression data with sqRT-PCR derived values.**

predictive classifier. In this small set, all six patients had sensitive tumours and were correctly classified by our predictive method.

Discussion

We obtained sufficient RNA from small core biopsy samples of human breast cancers, to assess patterns of gene expression in individual tumours and identified molecular profiles using gene expression patterns of human primary breast cancers to accurately predict sensitivity to docetaxel in women with primary breast cancer.

Gene expression patterns associated with docetaxel sensitivity and resistance are highly complex. In the past, investigators using single gene biomarkers to assess sensitivity and resistance to chemotherapy have seldom produced conclusive results. For example, in a breast cancer study the researchers did not note any correlation between commonly measured predictive and prognostic markers (HER-2, p53, p27, or epidermal growth factor receptor) and taxane sensitivity.[27] Reports of different cancer types have suggested that alterations in expression levels of β tubulin isoforms might represent an important and complex mechanism of taxane resistance.[28] We noted that overexpression of some β tubulin isoforms was associated with docetaxel resistance in some tumours, but not all. These results suggest that the patterns of gene expression for sensitivity and resistance are likely to involve multiple gene pathways, and that integration of many genes in these pathways leads to drug sensitivity and resistance. Our results lend support to the idea that assessment of expression of a few individual genes will not be powerful enough to untangle the heterogeneity of clinical breast cancers, but that patterns of expression of many genes could be successful in distinguishing between sensitive and resistant tumours.

A key point of this study was to focus on genes that could be reliably measured and to exclude those that were unlikely to be expressed in any sample. We did not design this study to discover specific genes for docetaxel response or resistance, but rather to identify patterns of many genes that could be used as a predictive test in patients with breast cancer. As a result, our analysis will have excluded some differential genes with low expression, some of which might be biologically interesting. For example, that spindle checkpoint dysfunction is an important cause of ANEUPLOIDY in human cancers has been suggested. The

serine-threonine kinase gene *STK6* (AURORA A)[28] might constitute a mechanism of spindle checkpoint dysregulation, and its amplification has been shown to predict resistance to taxanes.[29] Indeed, we did note differential expression between sensitive and resistant tumours—overexpression of *STK6* was about 1·4-fold higher in docetaxel-resistant tumours than in those that were sensitive to the drug (mean expression 506 and 695 in sensitive and resistant tumours, respectively; p=0·046). Nevertheless, this gene was not part of the 92-gene classifying list because of its overall low expression. This classifying list does not include all genes relevant to docetaxel sensitivity and resistance, but rather, identifies patterns of many genes that could be used as a predictive clinical test.

There is little information about the usefulness of gene expression arrays in human breast cancers.[19,30,32] Van't Veer and colleagues,[16] using printed oliogonucleotide microarrays, noted that gene expression profiles were more accurate predictors of outcome in a small set of 78 young women with node-negative breast cancer than more standard clinical and histological criteria. The same investigators subsequently validated this 70-gene classifier in a cohort of 295 patients, many of whom were not in the original study.[31] The signature of poor prognosis included genes regulating cell cycle, invasion, metastasis, and angiogenesis. Perou and colleagues[32] and Sorlie and colleagues[34] used cDNA arrays and identified distinct patterns of gene expression that were termed basal or luminal. These groups differed from each other with respect to clinical outcome.[18,31] Unlike these earlier publications that dealt with patient prognosis, our aim was to identify gene expression patterns that could predict response or resistance to docetaxel in patients with primary breast cancer.

Although breast cancers are highly heterogeneous, the classifying gene list gives some clues to the mechanisms of sensitivity and resistance in some tumours. In general, resistant tumours overexpressed genes associated with protein translation, cell cycle, and RNA transcription functions, whereas sensitive tumours overexpressed genes involved in stressor apoptosis, cytoskeleton, adhesion, protein transport, signal transduction, and RNA splicing or transport. Consistent with an apoptosis-induction mode of action for taxanes, sensitive tumours had higher expression of apoptosis-related proteins (eg, BAX, UBE2M, UBCH10, CUL1). DNA damage-related gene expression in docetaxel-sensitive tumours (overexpression of CSNK2B, DDB1, ABL, and underexpression of PRKDC) also seems to contribute to docetaxel sensitivity.

Furthermore, in sensitive tumours, overexpression of genes implicated in stress-related pathways was also noted, especially heat shock proteins. Overexpression of heat shock protein 27 (HSP27) has been associated with doxorubicin resistance in the MDA-MB-231 breast cancer cell line.[33] By contrast, the same investigators have shown that HSP27-overexpressing cell lines remain sensitive to docetaxel (Fuqua S, personal communication), suggesting that different non-cross-resistant agents could have different gene patterns of sensitivity and resistance. If true, then specific patterns of gene expression could be used as tools to choose between doxorubicin and docetaxel

In a leave-one-out cross-validation procedure, the classifier that included genes selected at the nominal value of p≤0·001 correctly classified tumours as sensitive or resistant in nearly 90% of cancers. Additionally, the predictive value of this classifier compares very favourably with that of oestrogen-receptor status, which is the only validated factor that can predict response to hormone treatment in breast cancer. Oestrogen-receptor status has a positive predictive value for response to hormone therapy of about 60%, and a negative predictive value of about 90%.[34] If about 70% of breast cancers are oestrogen-receptor positive, then sensitivity and specificity for hormone responsive and non-responsive tumours are about 93% and 50%, respectively, and the area under the ROC curve for oestrogen receptor is only about 0·72. The docetaxel classifier has positive and negative predictive values of 92% and 83%, respectively, and the area under the ROC curve of 0·96 (figure 3). Although these predictive values are likely to be slightly biased and have wide confidence intervals, these results suggest that classifiers based on gene expression would probably compare favourably with other clinically validated predictive markers.

Differences in RNA expression were confirmed by sqRT-PCR for a sample of genes. Furthermore, we have validated our classifier in an independent set of six consecutively treated patients, all of whom responded to treatment. Although the validation set is very small, it does lend support to the suggestion that gene expression arrays could be used to predict effectiveness of treatment.

This study shows that expression array technology can effectively and reproducibly classify tumours according to response or resistance to docetaxel chemotherapy. To ultimately define the molecular portrait of cancers sensitive or resistant to docetaxel, our results should be validated in a study with a large independent cohort of patients. Further patient recruitment and analysis will refine the gene list by which to classify tumours. This type of molecular profiling could have important clinical implications in defining the optimum treatment for an individual patient, thus reducing the use of unproductive treatments, unnecessary toxicity, and overall cost.

References

1 The Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomised trials involving 31 000 recurrences and 24 000 deaths among 75 000 women. *Lancet* 1992; 339: 1–15 (review).

2 The Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomised trials involving 31 000 recurrences and 24 000 deaths among 75 000 women. *Lancet* 1992; 339: 71–85.

3 The Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 351: 1451–67.

4   The Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 352: 930–42.

5   Hortobagyi GN. Docetaxel in breast cancer and a rationale for combination therapy. *Oncology* 1997; 11: 11–15.

6   Chan S, Friedrichs K, Noel D, et al. Prospective randomized trial of docetaxel versus doxorubicin in patients with metastatic breast cancer: the 303 Study Group. *J Clin Oncol* 1999; 17: 2341–54.

7   Nabholtz JM, Patterson A, Dirix L, et al. A phase III trial comparing docetaxel (T), doxorubicin (A) and cyclophosphamide (C) (TAC) to (FAC) as first line chemotherapy for patients with metastatic breast cancer. *Proc Am Soc Clin Oncol* 2001; 20: 22a.

8   Henderson IC Berry D A, Demetri GD, et al. Improved disease free survival and overall survival from the addition of sequential paclitaxel but not from escalation of doxorubicin in the adjuvant chemotherapy of patients with node-positive primary breast cancer. *Proc Am Soc Clin Oncol* 1998; 17: 101.

9   Aapro MS. Adjuvant therapy of primary breast cancer: a review of key finding s from the 8th international conference, St Gallen. *Oncologist* 2001; 6: 376–85.

10 Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* 1998; 16: 2672–85.

11 Mamounas EP. Preoperative doxorubicin plus cyclophosphamide followed by preoperative or postoperative docetaxel. *Oncology* 1997; 11(suppl 6): 37–40.

12 Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995; 270: 467–70.

13 Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Research* 1998; 58: 5009–13.

14 Lockhart DJ, Dong H, Byrne MC, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* 1996; 14: 1675–80.

15 Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR, Elkahloun AG. In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res* 1999; 59: 5656–61.

16 Perou CM, Jeffrey SS, van de Run M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci USA* 1999; 96: 9212–17.

17 Whitehead Institute/MIT centre for genome research. Primer 3. http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (accessed May 17, 2003).

18 van't Veer LJ, Dai H, van De Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002; 415: 530–36.

19 Schadt EE, Li C, Ellis B, Wong WH. Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J Cell Biochem Suppl* 2001; 37 (suppl 37): 120–25.

20 Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci USA* 2001; 98: 31–36.

21 Li C, Wong WH. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol* 2001; 2: research0032.1–0032.11.

22 Simon R, Peng A. http://linus.nci.nih.gov/BRB-ArrayTools.html (accessed May 17, 2003).

23 Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. *J Comput Biol* 2002; 9: 505–11.

24 Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. *Proc Natl Acad Sci USA* 2002; 99: 6562–66.

25 Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J Natl Cancer Inst* 2003; 95: 14–18.

26 NCBI. Gene expression omnibus: accession numbers GSE 349 (resistant samples) and GSE 350 (sensitive samples). www.ncbi.nlm.nih.gov/geo (accessed May 17, 2003).

27 Van Poznak C, Tan L, Panageas KS, et al. Assessment of molecular markers of clinical sensitivity to single-agent taxane therapy for metastatic breast cancer. *J Clin Oncol* 2002; 20: 2319–26.

28 Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* 1999; 17: 1061–70.

29 Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell* 2003; 3: 51–62.

30 van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002; 347: 1999–2009.

31 Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; 98: 10869–74.

32 Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000; 406: 747–52.

33 Hansen RK, Parra I, Lemieux P, Oesterreich S, Hilsenbeck SG, Fuqua SA. Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. *Breast Cancer Res Treat* 1999; 56: 187–96.

34 Osborne CK, Yochmowitz MG, Knight WA, 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980; 46 (suppl 12): 2884–88.

Expression Patterns for Docetaxel Sensitivity and Resistance

Jenny C. Chang

Breast Center at Baylor College of Medicine, Houston, Texas

Breast cancer is the most common malignancy afflicting women from Western cultures. It has been estimated that more than 40,000 women will die of this disease in the United States alone. Developments in breast cancer molecular and cellular biology research have brought us closer to understanding the genetic basis of this disease. Unfortunately, this information has yet to be incorporated into the routine diagnosis and treatment of breast cancer in the clinic. Recent advancements in microarray technology hold the promise of further increasing our understanding of the complexity and heterogeneity of this disease and providing new avenues for the prognostication of breast cancer outcomes. The most recent application of microarray genomic technologies in studying breast cancer, in particular prediction of response to docetaxel,[1] will be the focus of this review.

Developments in breast cancer molecular and cellular biology research . . . has yet to be incorporated into the routine diagnosis and treatment of breast cancer in the clinic.

Docetaxel in Breast Cancer

Optimal systemic treatment (adjuvant therapy) after breast cancer surgery is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients.[2-4] However, while estrogen receptor status predicts for response to hormonal treatments, there are no clinically useful predictive markers for chemotherapy response. All eligible women are therefore treated in the same manner even though de novo drug resistance will result in treatment failures in many breast cancer patients. The taxanes, docetaxel (Taxotere®) and paclitaxel (Taxol®), are a new class of antimicrotubule agents that are more effective than older drugs like the anthracyclines,[5-7] although clinical trials with taxanes and anthracyclines in combination show that only a small subset of patients benefit from the addition of taxanes.[8,9] Currently, there are no methods available to distinguish those patients who are likely to respond to taxanes from those who are not, and given the accepted practice of prescribing adjuvant treatment to most patients even if the average expected benefit is low, the a priori selection of appropriate patients most likely to benefit from adjuvant taxane therapy would represent a major advance in the clinical management of breast cancer today.[8,9] A major impediment to study predictors of therapeutic efficacy in the adjuvant setting is the lack of surrogate markers for survival and, consequently, large numbers of patients with long-term follow-up are needed to conduct these studies.

We therefore set out to identify gene expression patterns in primary breast cancer specimens that might predict response to taxanes. Neoadjuvant chemotherapy (treatment before primary surgery) allows for sampling of

the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment.[10,11] With the advent of high-throughput quantitation of gene expression, it is now

> *Optimal systemic treatment after breast cancer surgery is the most crucial factor in reducing mortality in women with breast cancer.*

possible to assess thousands of genes simultaneously to identify expression patterns in different breast cancers that might correlate with and thereby predict excellent clinical response to treatment.[12-16] Hence, neoadjuvant chemotherapy provides an ideal platform to rapidly discover predictive markers of chemotherapy response.

Study Design

Patients with locally advanced breast cancer (primary cancers >4 cm, or with clinically evident axillary metastases) were considered for a phase II study with neoadjuvant docetaxel. Clinical staging and size of primary tumor were recorded at the start of treatment, at each cycle, and after completion of 4 cycles of chemotherapy. Tumor size (product of the two largest perpendicular diameters) meas-

ured before and after 4 cycles of neoadjuvant chemotherapy was used to compute the percentage of residual disease. The median residual disease was then calculated, and this degree of response was then used to divide the cancers into two groups of sensitive and resistant categories of approximately equal numbers before gene expression analysis.

Core biopsies of the primary cancers were undertaken before administration of single-agent docetaxel as neoadjuvant treatment. Docetaxel at 100 mg/m^2 was given every 3 weeks for a total of 4 cycles, and clinical response was assessed after the fourth cycle at 12 weeks.

Total RNA was isolated from the frozen core biopsy specimens according to protocols recommended by Affymetrix (Santa Clara, CA) for GeneChip® experiments. Each core biopsy yielded 3 to 6 micrograms of total RNA. After RNA recovery, double-stranded cDNA was then synthesized. Reverse transcription was carried out according to protocols recommended by Affymetrix (Santa Clara, CA) using commercially available buffers and proteins (Invitrogen Corporation, Carlsbad, CA). Biotin labeling and approximately 250-fold linear amplification followed phenol-chloroform cleanup of the reverse transcription reaction product and was carried out by in vitro transcription. From each biopsy 15 micrograms of labeled cRNA was then hybridized onto the Affymetrix U95Av2 GeneChip®. The Affymetrix U95Av2 GeneChip® comprises about 12,625 probe sets.

Results

Before treatment, the median tumor size was 8 cm (range 4 to 30 cm). Prior to gene expression analysis,

we defined sensitivity and resistance based on the percentage of residual disease after treatment. We first determined that the median residual disease after chemotherapy was 30%. We then arbitrarily defined sensitive tumors as those with ≤25% residual disease and resistant tumors as those with >25% residual disease, as this cut off divides the number of patients almost equally into two groups for statistical comparison.

Of these 24 patients, 11 were sensitive (46%) to docetaxel and 13 were resistant (54%). Of the sensitive tumors, 5 patients (5/11, 45%) had minimal residual disease (<10% residual tumor), while of the resistant tumors, 7 patients had residual tumors ≥60% (7/13, 54%), and 3 of these women (3/13, 23%) had residual tumors that were 100% or greater of baseline.

> *Currently, there are no methods available to distinguish those patients who are likely to respond to taxanes from those who are not.*

Selection of discriminatory genes. We compared the expression data in the sensitive and the resistant tumors to identify genes significantly differentially expressed between the two groups. We first selected a subset of candidate genes by filtering on signal

intensity to eliminate genes with uniformly low expression or genes whose expression did not vary significantly across the samples, retaining 1,628 genes. After log transformation, a t test was used to select discriminatory genes. To evaluate the possibility of spurious results due to multiple comparisons, we performed a global permutation test that evaluates the statistical probability of obtaining the observed number of differentially expressed genes (or more) by chance alone. T tests with nominal P values of 0.001, 0.01, and 0.05 selected, respectively, 92, 300, and 551 genes as "differentially expressed." The probability that these numbers of genes would be selected by chance alone was estimated to be 0.0015, 0.001, and <0.001, respectively.

Functional classification of discriminatory genes. The 92 genes classed as most significantly "differentially expressed" at nominal P value <0.001 showed 4.2- to 2.6-fold decreases or 2.5- to 15.7-fold increases in expression in resistant versus sensitive tumors. Functional classes of these differentially expressed genes included stress/apoptosis (21%), cell adhesion/cytoskeleton (16%), protein transport (13%), signal transduction (12%), RNA transcription (10%), RNA splicing/transport (9%), cell cycle (7%), and protein translation (3%); the remainder (9%) had unknown functions.

Discussion

This study was designed to identify and confirm patterns of gene expression associated with docetaxel sensitivity or resistance. From human breast cancers, sufficient RNA was obtained from small core biopsies to assess gene expression patterns in individual tumors. To the best of our knowledge, this is the first study to have identified

We set out to identify gene expression patterns in primary breast cancer specimens that might predict response to taxanes.

molecular profiles using gene expression patterns of human primary breast cancers which may be useful to accurately predict response or lack of response to chemotherapy. The results of this study suggest that molecular profiling has the potential to accurately predict docetaxel response in primary breast cancer patients. ∎

References

1. J Chang, E Wooten, A Tsimelzon, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet.* 2003;362:362-369.
2. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Early Breast Cancer Trialists' Collaborative Group. *Lancet.* 1992;339:1-15, 71-85.
3. Tamoxifen for early breast cancer: an overview of the randomised trials. The Early Breast Cancer Trialists' Collaborative Group. *Lancet.* 1998;351:1451-1467.
4. Polychemotherapy for early breast cancer: an overview of the randomised trials. The Early Breast Cancer Trialists' Collaborative Group. *Lancet.* 1998;352:930-942.
5. Hortobagyi GN. Docetaxel in breast cancer and a rationale for combination therapy. *Oncology.* 1997;11:11-15.
6. Chan S, Friedrichs K, Noel D, et al. Prospective randomized trial of docetaxel versus doxorubicin in patients with metastatic breast cancer. The 303 Study Group. *J Clin Oncol.* 1999;17:2341-2354.
7. Nabholtz JM, Patterson A, Dirix L, et al. A phase III trial comparing docetaxel (T), doxorubicin (A) and cyclophosphamide (C) (TAC) to (FAC) as first line chemotherapy for patients with metastatic breast cancer. *Proc Am Soc Clin Oncol.* 2001;20:22a.
8. Henderson IC, Berry D, Demetri G, et al. Improved disease free survival and overall survival from the addition of sequential paclitaxel but not from the escalation of doxorubicin dose level in the adjuvant chemotherapy of patients with node-positive primary breast cancer. *Proc Am Soc Clin Oncol.* 1998;17:101.
9. Aapro MS. Adjuvant therapy of primary breast cancer: a review of key findings from the 7th international conference, St. Gallen, February 2001. *Oncologist.* 2001;6:376-385.
10. Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol.* 1998;16:2672-2685.
11. Mamounas EP. Preoperative doxorubicin plus cyclophosphamide followed by preoperative or postoperative docetaxel. *Oncology.* 1997;11(suppl 6):37-40.
12. Schena M, Shalon D, Davis RW, et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science.* 1995;270:467-470.
12. Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Res.* 1998;58:5009-5013.
13. Lockhart DJ, Dong H, Byrne MC, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol.* 1996;14:1675-1680.
14. Sgroi DC, Teng S, Robinson G, et al. In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res.* 1999;59:5656-5661.
15. Perou CM, Jeffrey SS, van de Rijn M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Nat Acad Sci U S A.* 1999;96:9212-9217.
16. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature.* 2002;415:530-536.
17. Schadt EE, Li C, Ellis B, et al. Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J Cell Biochem Suppl.* 2001;suppl 37:120-125.
18. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. *Proc Natl Acad Sci U S A.* 2001;98:31-36.

19. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol.* 2001;2:RESEARCH0032. Epub 2001 Aug 03.

20. Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. *J Comput Biol.* 2002;9:505-511.

21. Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. *Proc Natl Acad Sci U S A.* 2002;99:6562-6566.

22. Simon R, Radmacher MD, Dobbin K, et al. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J Natl Cancer Inst.* 2003;95:14-18.

23. McNeil BJ, Hanley JA. Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. *Med Decis Making.* 1984;4:137-150.

24. Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell.* 2003;3:51-62.

25. Van Poznak C, Tan L, Panageas KS, et al. Assessment of molecular markers of clinical sensitivity to single-agent taxane therapy for metastatic breast cancer. *J Clin Oncol.* 2002;20:2319-2326.

26. Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol.* 1999;17:1061-1070.

27. van de Vijver MJ, He YD, van 't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002;347:1999-2009.

28. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A.* 2001;98:10869-10874.

29. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature.* 2000;406:747-752.

30. Hansen RK, Parra I, Lemieux P, et al. Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. *Breast Cancer Res Treat.* 1999;56:187-196.

31. Osborne CK, Yochmowitz MG, Knight WA 3rd, et al. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer.* 1980;46(12 suppl):2884-2888.

Correspondence Address: Dr. Jenny C. Chang, Breast Center at Baylor College of Medicine, 1 Baylor Plaza, BCM 600, Houston, TX 77030; phone: (713) 798-1905; fax: (713) 798-1642; e-mail: jcchang@breastcenter.tmc.edu

CANCER CONFERENCE
HIGHLIGHTS

Hierarchical Clustering of Genes Correlated With Docetaxel Response

AN UPDATE ON TAXANE-BASED THERAPY FOR BREAST CANCER

Selected Presentations From the
39th Annual Meeting of the American Society
of Clinical Oncology
Chicago, Il.
May 31-June 3, 2003

GENE EXPRESSION PATTERNS FOR DOCETAXEL SENSITIVITY AND RESISTANCE IN PATIENTS WITH LOCALLY ADVANCED BREAST CANCER

Jenny C. Chang, MD
Assistant Professor
Breast Center at Baylor
College of Medicine
Houston, TX

Introduction

Breast cancer is the most common malignancy afflicting women from Western cultures. It has been estimated that approximately 211,000 women will be diagnosed with breast cancer in 2003 in the United States alone, and distressingly, each year approximately 40,000 women will die of this disease.[1] Developments in breast cancer molecular and cellular biology research have brought us closer to understanding the genetic basis of this disease. Unfortunately, this information has not yet been incorporated into the routine diagnosis and treatment of breast cancer in the clinic. Recent advancements in microarray technology hold the promise of further increasing our understanding of the complexity and heterogeneity of this disease and of providing new avenues for the prognostication and prediction of breast cancer outcomes. The most recent application of microarray genomic technologies in studying breast cancer, in particular prediction of response to chemotherapy, will be the focus of this review.[2]
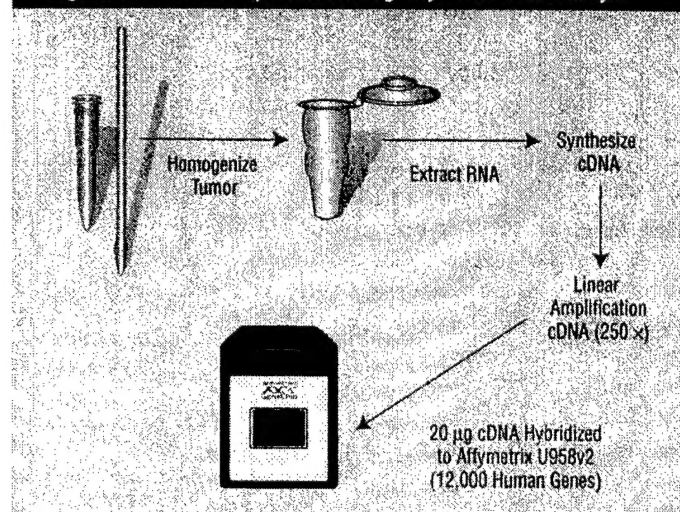
Gene Expression Patterns as Predictors of Response to Docetaxel in Breast Cancer

Optimal systemic treatment (adjuvant therapy) after breast cancer surgery is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients.[3-5] However, while estrogen receptor status predicts for response to hormonal treatments, there are no clinically useful predictive markers for chemotherapy response. All eligible women are therefore treated in the same manner, even though de novo drug resistance will result in treatment failures in many breast cancer patients. The taxanes docetaxel (Taxotere®) and paclitaxel (Taxol®) are a new class of antimicrotubule agents that are more effective than older drugs like the anthracyclines,[6-8] although clinical trials with taxanes and anthracyclines in combination show that only a small subset of patients benefit from the addition of taxanes.[9,10] Currently, there are no methods available to distinguish those patients who are likely to respond to taxanes

from those who are not, and given the accepted practice of prescribing adjuvant treatment to most patients even if the average expected benefit is low, the a priori selection of appropriate patients most likely to benefit from adjuvant taxane therapy would represent a major advance in the clinical management of breast cancer today.[9,10] A major impediment to study predictors of therapeutic efficacy in the adjuvant setting is the lack of surrogate markers for survival and, consequently, large numbers of patients with long-term follow-up are needed to conduct these studies.
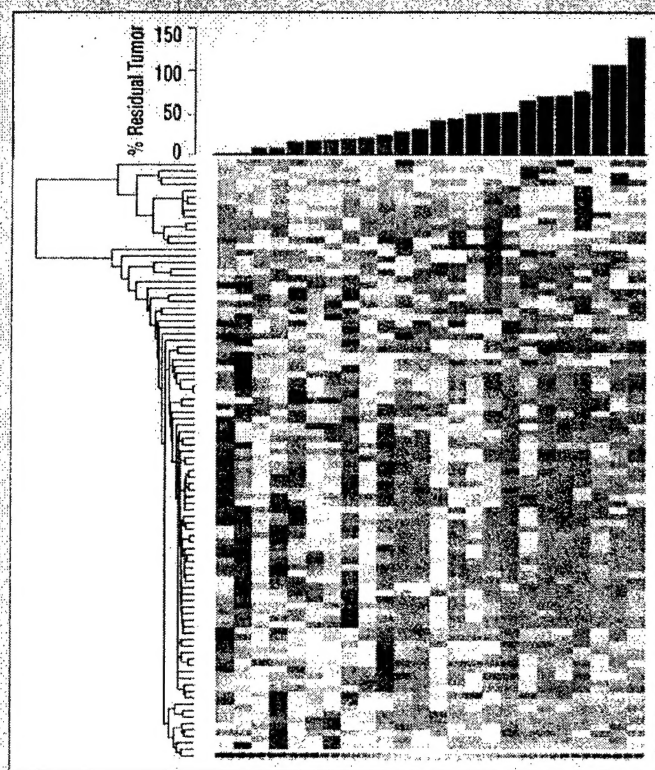
We therefore set out to identify gene expression patterns in primary breast cancer specimens that might predict response to taxanes. Neoadjuvant chemotherapy (treatment before primary surgery) allows for sampling of the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment.[11,12] This clinical tumor response to neoadjuvant chemotherapy has been shown to be a valid surrogate marker of survival, with better outcome in those patients whose tumors regress significantly after neoadjuvant chemotherapy compared to those with modest response or clinically obvious chemotherapy-resistant disease.[11,12] With the advent of high-throughput quantitation of gene expression, it is now possible to assess thousands of genes simultaneously to identify expression patterns in different breast cancers that might correlate with and thereby predict excellent clinical response to treatment.[13-17] These profiles have a great potential to penetrate the genetic heterogeneity of this disease and prioritize different treatment

Figure 1   Gene Expression Using Affymetrix cDNA Arrays

Homogenize Tumor → Extract RNA → Synthesize cDNA → Linear Amplification cDNA (250 ×) → 20 μg cDNA Hybridized to Affymetrix U958v2 (12,000 Human Genes)

Figure 2 — Hierarchical Clustering of Genes Correlated With Docetaxel Response

Sensitive tumors are defined as ≤ 25% residual disease (shown as blue bars), and resistant tumors are defined as > 25% residual disease (shown as red bars). The expression levels are shown in red (expression levels above the mean for the gene) and blue (levels below the mean for the gene). The color scale (see bottom of figure) ranges from 3 standard deviations (or more) below the mean (darkest blue) to 3 standard deviations above the mean (darkest red).

strategies based on their likelihood of success in individual patients. Hence, neoadjuvant chemotherapy provides an ideal platform to rapidly discover predictive markers of chemotherapy response.

Study Design

Eligibility. Patients with locally advanced breast cancer (primary cancers > 4 cm or with clinically evident axillary metastases) were considered for a phase II study with neoadjuvant docetaxel.[18] In brief, the inclusion criteria were (1) age > 18 years, (2) a diagnosis of breast cancer confirmed by core needle biopsy, (3) premenopausal status accompanied by appropriate contraception, (4) adequate performance status, and (5) adequate liver and kidney function test results (all within 1.5 times the institution's upper limit of normal). Exclusion criteria included (1) severe underlying chronic illness or disease and (2) treatment with other chemotherapeutic drugs while on the study.

Clinical staging and size of the primary tumor was recorded at the start of treatment, at each cycle, and after completion of 4 cycles of chemotherapy. Tumor size (product of the 2 largest perpendicular diameters) measured before and after 4 cycles of neoadjuvant chemotherapy was used to compute the percentage of residual disease. The median residual disease was then calculated, and this degree of response was then used to divide the cancers into 2 groups of sensitive and resistant categories of approximately equal

numbers before gene expression analysis. Core biopsies of the primary cancers were undertaken before administration of single-agent docetaxel as neoadjuvant treatment.

Treatment. Docetaxel 100 mg/m² was given every 3 weeks for a total of 4 cycles, and clinical response was assessed after the fourth cycle (at 12 weeks). Patients continued on neoadjuvant chemotherapy through the full 4 cycles unless there was clear documentation of progressive disease, defined as an increase in tumor size of > 25%. Primary surgery and standard adjuvant therapy were then administered following completion of neoadjuvant docetaxel. In order to maximize the likelihood of obtaining sufficient tissue, approximately 6 core biopsies were taken. Two to three core biopsy specimens were immediately transferred for snap freezing at −80°C for cDNA array analysis.

Gene Profiling Methods. Total RNA was isolated from the frozen core biopsy specimens according to protocols recommended by Affymetrix (Santa Clara, CA) for GeneChip® experiments (Figure 1). Each core biopsy yielded 3-6 μg of total RNA. After RNA recovery, double-stranded cDNA was then synthesized. Reverse transcription was performed according to protocols recommended by Affymetrix using commercially available buffers and proteins (Invitrogen Corporation, Carlsbad, CA). Biotin labeling and approximately 250-fold linear amplification followed phenol:chloroform cleanup of the reverse-transcription reaction product and was performed using in vitro transcription. From each biopsy, 15 μg of labeled cRNA was then hybridized onto the Affymetrix U95Av2 GeneChip. The Affymetrix U95Av2 GeneChip comprises approximately 12,625 probe sets, each containing approximately 16 perfect-match and corresponding mismatch 25-mer oligonucleotide probes, representing sequences (genes), most of which have been characterized in terms of function or disease association.

Results

Before treatment, the median tumor size was 8 cm (range, 4-30 cm). Prior to gene expression analysis, we defined sensitivity and resistance based on the percentage of residual disease after treatment. We first determined that the median residual disease after chemotherapy was 30%. We then arbitrarily defined sensitive tumors as those with ≤ 25% residual disease and resistant tumors as those with > 25% residual disease, as this cutoff divides the numbers of patients almost equally into 2 groups for statistical comparison.

Of the 24 patients, 11 (46%) were sensitive to docetaxel and 13 (54%) were resistant. Of the 11 patients with sensitive tumors, 5 (45%) had minimal residual disease (< 10% residual tumor), while of the 13 patients with resistant tumors, 7 (54%) had residual tumors ≥ 60%. Three of these women (23%) had residual tumors that were ≥ 100% of baseline.

Selection of Discriminatory Genes

We compared the expression data in the sensitive and resistant tumors to identify genes significantly differentially expressed between the 2 groups (Figure 2). We first selected a subset of candidate genes by filtering on signal intensity to eliminate genes with uniformly low expression or genes whose expression did not vary significantly across the samples, retaining 1628 genes. After log transformation, a *t* test was used to select discriminatory genes. To evaluate the possibility of spurious results due to multiple comparisons, we performed a global permutation test, which evaluates the statistical probability of obtaining the observed number of differentially expressed genes (or more) by chance alone. The *t* tests with nominal

Table 1	Functional Classes of 92 Genes With Differential Expression Between Docetaxel-Sensitive and Docetaxel-Refractory Tumors
Stress/Apoptosis	21%
Cell Adhesion/Cytoskeleton	16%
Protein Transport	13%
Signal Transduction	12%
Transcription	10%
Splicing Transport	9%
Cell Cycle	7%
Protein Translation	3%

Nine percent of the genes differentially expressed between docetaxel-sensitive and docetaxel-resistant tumor samples have unknown function.

P values of .001, .01, and .05 selected, respectively, 92, 300, and 551 genes as differentially expressed. The probability that these numbers of genes would be selected by chance alone was estimated to be .0015, .001, and < .001, respectively.

Functional Classification of Discriminatory Genes. The 92 genes classified as most significantly differentially expressed at a nominal *P* value < .001 showed 2.6- to 4.2-fold decreases or 2.5- to 15.7-fold increases in expression in resistant versus sensitive tumors. Functional classes of these differentially expressed genes included stress/apoptosis (21%), cell adhesion/cytoskeleton (16%), protein transport (13%), signal transduction (12%), RNA transcription (10%), RNA splicing transport (9%), cell cycle (7%), and protein translation (3%) (Table 1).

Discussion

This study was designed to identify and confirm patterns of gene expression associated with docetaxel sensitivity or resistance. From human breast cancers, sufficient RNA was obtained from small core biopsies to assess gene expression patterns in individual tumors. To the best of our knowledge, this is the first study to have identified molecular profiles using gene expression patterns of human primary breast cancers to accurately predict response or lack of response to chemotherapy. The results of this study suggest that molecular profiling has the potential to accurately predict docetaxel response in primary breast cancer patients.

References

1. Jemal A, Murray T, Samuels A, et al. Cancer statistics, 2003. *CA Cancer J Clin* 2003; 53:5-26.
2. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel (Taxotere™) in breast cancer patients. *Lancet.* In press.
3. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* 1992; 339:71-85.
4. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 351:1451-67.
5. Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998; 352:930-42.
6. Hortobagyi G. Docetaxel in breast cancer and a rationale for combination therapy. *Oncology (Huntingt)* 1997; 11(suppl 6):11-5.
7. Chan S, Friedrichs K, Noel D, et al. Prospective randomized trial of docetaxel versus doxorubicin in patients with metastatic breast cancer. The 303 Study Group. *J Clin Oncol* 1999; 17:2341-54.
8. Nabholtz JA, Paterson A, Dirix L, et al. A phase III randomized trial comparing docetaxel (T), doxorubicin (A) and cyclophosphamide (C) (TAC) to FAC as first line chemotherapy (CT) for patients (pts) with metastatic breast cancer (MBC). *Proc Am Soc Clin Oncol* 2001; 20:22a (abstract 83).
9. Henderson IC, Berry DA, Demetri GD, et al. Improved outcomes from adding sequential paclitaxel but not from escalating doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. *J Clin Oncol* 2003; 21:976-83.
10. Aapro MS. Adjuvant therapy of primary breast cancer: a review of key findings from the 7th international conference, St. Gallen, February 2001. *Oncologist* 2001; 6:376-85.
11. Fisher B, Bryant J, Wolmark N, et al. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* 1998; 16:2672-85.
12. Mamounas EP. NSABP Protocol B-27. Preoperative doxorubicin plus cyclophosphamide followed by preoperative or postoperative docetaxel. *Oncology (Huntingt)* 1997; 11(suppl 6):37-40.
13. Schena M, Shalon D, Davis RW, et al. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995; 270:467-70.
14. Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Res* 1998; 58:5009-13.
15. Lockhart DJ, Dong H, Byrne MC, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 1996; 14:1675-80.
16. Sgroi DC, Teng S, Robinson G, et al. In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res* 1999; 59:5656-61.
17. Perou CM, Jeffrey SS, van de Rijn M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci U S A* 1999; 96:9212-7.
18. Chang JCN, Wooten EC, Tsimelzon A, et al. Gene expression patterns for de novo and acquired docetaxel resistance in patients with locally advanced breast cancer. *Proc Am Soc Clin Oncol* 2003; 22:9 (abstract 32).